RE-EXAMINATION OF AN INHIBITOR OF REGENERATION IN TUBULARIA

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In the marine hydroid *Tubularia* the presence of hydranth structures has been thought to prevent the development of new hydranths in nearby stem tissue. Two preparations have been made from adult hydranths which inhibited the regeneration of new hydranths on isolated stem segments. One of these (*inhibitor water,* Rose and Rose, 1941) was made by agitating adult hydranths in aerated sea water for from 12 to 24 hours, while the other (*hydranth extract,* Tardent, 1955) was found in the supernatants of homogenates of adult hydranths. These inhibitors of regeneration were specific to hydranth tissue in that they were not obtained when stems were treated in the same manner. They have been compared (Tweedell, 1958) and found to differ in a number of properties. The regeneration-inhibiting substances in inhibitor water have been considered by a number of authors to represent the substances normally responsible for physiological dominance in *Tubularia,* and inhibitor water has been employed by Steinberg (1954) in an experiment to indicate the mechanism of physiological dominance.

In the present investigation it was found that active inhibitor water could not be prepared in the absence of bacterial growth, and as a consequence a re-examination of this inhibitor was undertaken.

Materials and Methods

Freshly-collected *Tubularia crocea,* provided by the Supply Department of the Marine Biological Laboratory, was used in all experiments. Sea water was filtered through paper shortly before use.

In preparing inhibitor water, an attempt was made to use methods comparable to those used by previous workers (*cf.* Tweedell, 1958). Populations of adult hydranths with 5 mm. of stem attached were isolated and washed thoroughly, and then aerated in sea water for 24 hours at 17–22° C. After aeration, the hydranths and debris were removed by filtration and the preparation was tested for its effect on regeneration.

The bacterial population was estimated subjectively in early experiments by turbidity and microscopical examination, and in later experiments was determined using a Petroff-Hauser bacteria-counting slide. The bacterial population of filtered sea water was found to be approximately 10^5 per ml., which is too low for accurate estimation with a counting slide. It was assumed that no bacterial proliferation had occurred during the preparation of any given solution if the bacterial population did not exceed this order of magnitude. It should be cautioned that if mature male hydranths are used to prepare inhibitor water, turbidity may in part result from the release of large numbers of sperm into the water.

When it was desired to remove bacteria, the preparations were filtered through an HA millipore filter (Millipore Filter Corp., Watertown, Mass.) or centrifuged for 5 minutes at 30,000 g. Bacterial growth was prevented by the addition of antibiotics. Penicillin and streptomycin were used at 100–125 μg./ml.; sulfadiazine was used at about 0.001 per cent (or saturation in sea water). At these concentrations, and in the cases of penicillin and streptomycin even at four-fold higher concentrations, the antibiotics did not have any significant effect on the rate or course of regeneration.

The solutions to be described were tested immediately after preparation for their effect on the regeneration of freshly-cut, 7-mm. stem segments. Virtually all of the stems in control groups regenerated, although as is usual with *Tubularia* there was a considerable variation in the rate, even within a single group. A preparation was considered to have inhibited regeneration if, during the time required for the complete regeneration of the controls (emergence), all or a significant fraction of the experimental group either disintegrated or healed but did not begin regeneration. Stems in inhibitor water which regenerated were usually but not always retarded.

Results

Populations of 1.5–2 hydranths per ml. aerated in sea water regularly produced an inhibitor water which completely prevented regeneration. Occasional batches of inhibitor water prepared at these or lower hydranth densities were inactive, while populations of more than two hydranths per ml. usually gave a preparation which caused disintegration of the stem tissue. However, considerable variability was found in the activity of preparations made at the same hydranth densities and under the same conditions (temperature, time, etc.), suggesting that some factor other than those controlled was involved.

A number of observations suggested that the activity of inhibitor water was due to bacterial growth. Preparations became quite turbid during the course of aeration, and the condition of the hydranths deteriorated rapidly. The solution developed a putrid odor. Hydranths killed by exposure to 30° C. for 15 minutes rapidly disintegrated, but nevertheless produced active inhibitor water. When active preparations were examined microscopically, a large, heterogeneous population of bacteria was found. Removal of these bacteria often resulted in a reduction but never in an elimination of the inhibitory activity of a preparation.

An estimate of the amount of bacterial growth which occurs in inhibitor water preparations was obtained by preparing a series of 5 inhibitor waters at a density of 1.5 hydranths per ml. and making counts with a bacteria counting slide at the beginning and end of aeration. In this series, the bacterial density increased during aeration from about 3×10^5 bacteria per ml. to about 10^8 bacteria per ml. Bacteria were removed by centrifugation and each preparation tested for its effect on the regeneration of 10 stems. The results are given in Table I. The increase in bacterial number represents a minimum of 9 generations of bacterial growth. It should be noted, however, that counts made at the beginning of aeration do not include bacteria which are present in the hydranths and are released into the water during aeration as the hydranths disintegrate.

In order to determine whether or not hydranths could produce active inhibitor water in the absence of bacterial growth, hydranths were agitated in sea water

containing antibiotics at concentrations sufficient to maintain bacteriostasis. The results of three of the experiments with penicillin and streptomycin are given in Table II. In the first two experiments shown (A and B), the amount of bacterial growth was estimated subjectively. No detectable bacterial growth occurred in any of the preparations in experiment A, and in spite of the fact that the hydranth density was more than twice that necessary to produce complete inhibition in the absence of antibiotics, the stems in both experimental groups all regenerated at the same rate as the control stems. In experiment B, the hydranth density was over four times that necessary to produce complete inhibition without antibiotics. The preparation agitated at this hydranth density with antibiotics (B4) produced some delay in the rate of regeneration. This delay may have been due to the cytolysis of some of the hydranth tissue releasing the same substances present in hydranth extracts (see discussion).

These experiments indicate that in the presence of penicillin and streptomycin in concentrations which suppress bacterial growth, inhibitor water cannot be collected. However, it may be argued (Tweedell, 1958) that the antibiotics used either prevent the hydranths from producing an inhibitor of regeneration or destroy this inhibitor as it is produced. Three observations appear to exclude these

TABLE I

The number of bacteria present in five similar preparations of inhibitor water and the effect of these preparations on regeneration. Observations were made at intervals for 4.3 days

Preparation	Bacteria/ml. $\times 10^8$	Effect of preparation on stems
Control	0.0	10/10 emerged within 2.4 days
1	2.3	10/10 did not begin regeneration
2	3.3	10/10 did not begin regeneration
3	3.7	10/10 disintegrated within 3.6 days
4	5.5	10/10 disintegrated within 1.4 days
5	7.4	10/10 disintegrated within 1.0 day

alternatives. (1) That penicillin and streptomycin do not destroy the inhibitors was demonstrated by experiments in which bacteria were removed by centrifugation and these antibiotics added to inhibitor water after preparation. In one such experiment, there was no measurable reduction in the activity of the preparation when antibiotics were added (Table II, C3); in another (not listed) there was a slight reduction but not an elimination of the inhibition produced by the preparation (similar to that observed in other preparations when they were sterile filtered). (2) Particularly important are three experiments with penicillin and streptomycin and one with sulfadiazine in which bacterial growth occurred in the preparations even though antibiotic was added at the beginning of aeration. Presumably bacteria resistant to the antibiotics used developed in these preparations. In these cases, the preparations inhibited regeneration in proportion to the amount of bacterial growth which occurred in them (*e.g.*, Table II, C5, 6), showing that, in spite of the antibiotics, if bacterial growth occurred, an active regeneration inhibitor was produced. (3) Three antibiotics—penicillin, streptomycin and sulfadiazine—differing greatly in chemical structure and presumed mode of action, were used alone or in pairs to maintain bacteriostasis. Regardless of which antibiotic was used, if bacterial growth was prevented the preparation failed to inhibit regeneration.

To see if hydranths were a necessary component of the system, experiments were done in which bacterial growth was allowed to occur in sea water in the absence of hydranths. Dilute proteose-peptone solutions in sea water, aerated for 24 hours, and then sterilized by millipore filtration followed by the addition of antibiotic, were potent inhibitors of regeneration, while control solutions in which

TABLE II

Selected experiments which illustrate the activity of inhibitor water prepared with penicillin and streptomycin. Bacterial density was either estimated (number represented by pluses in the table) or counted directly using a bacteria counting slide (represented by number per ml.). Abbreviations: pen., penicillin; strep., streptomycin

Experiment	Components added to sea water	Bacteria per ml.	Stems regenerated vs. total	Mean time of emergence in days
A1	Pen. and strep.	—	10/10	2.5
2	4 hydranths/ml. + pen. and strep.	—	10/10	2.6
3	4 hydranths/ml. + pen. and strep.	—	10/10	2.5
B1	None	—	10/10	2.3
2	8 hydranths/ml.	+++	0/10	—
3	Pen. and strep.	—	10/10	2.4
4	8 hydranths/ml. + pen. and strep.	—	10/10	3.1
C1	None	ca. 10^5	10/10	2.4
2	2 hydranths/ml.	5×10^8	0/10	—
3	2 hydranths/ml., pen. and strep. added after aeration*		0/10	—
4	Pen. and strep.	$< 10^5$	10/10	2.3
5	2 hydranths/ml. + pen.	2×10^7	5/10	4.8
6	2 hydranths/ml. + strep.	1×10^8	2/10	2.3
7	0.1% proteose peptone + pen. and strep.	ca. 10^5	10/10	3.1
8	0.1% proteose peptone, pen. and strep. added after aeration	3×10^8	0/10	—

* Penicillin and streptomycin were added to a portion of solution C2.

bacterial growth was prevented by the addition of antibiotic at the beginning of aeration, at most, slightly retarded regeneration (*e.g.*, Table II, C7, 8; compare C1, 2).

To make certain that the inhibition produced as a result of bacterial growth was not dependent on the presence of specific bacteria, preparations were made

using *Escherichia coli*. Cultures were grown in a minimal medium (Davis and Mingioli, 1950) from a small inoculum to 10^9 cells per ml. The bacteria were removed by centrifugation, and the used medium diluted 1:5 in sea water containing antibiotic. Such a preparation completely inhibited regeneration, while control stems placed in a 1:5 dilution of sterile minimal medium with antibiotic regenerated normally.

From these data it is clear that the activity of inhibitor water can be explained on the basis of the bacterial growth which occurs in the medium, and that no other inhibitors can be collected when bacteriostasis is maintained with antibiotics.

TABLE III

Summary of all experiments which indicate that inhibitor water is a by-product of bacterial growth. Refer to the text for explanations of each experiment. Abbreviations: pen., penicillin; strep., streptomycin; sulfa., sulfadiazine

Components added to sea water before aeration	Number of experiments	Bacterial growth	Inhibition of regeneration
None	17	−	−
Hydranths	17	+	+
Hydranths*	6	+	+
Hydranths**	2	+	+
Heat-killed hydranths	4	+	+
Pen., strep., or sulfa.	14	−	−
Hydranths + pen., strep., or both pen. and strep.	7	−	−
	3	+	+
Hydranths + sulfa.	2	−	−
	1	+	+
Stem lengths	2	−	−
Proteose peptone, pen. and strep.	3	−	−
Proteose peptone**	4	+	+

* Preparation sterile filtered after aeration.

** Preparation centrifuged after aeration, penicillin and streptomycin added to the supernatant.

As an argument for the specific role of hydranth structures in producing inhibitor water it has been noted that a population of stems, aerated in sea water, does not produce an inhibitor (Tweedell, 1958). After cutting, the ends of a stem rapidly heal and secrete a thin layer of perisarc, so that very soon a cut stem is entirely covered with chitin. Since no tissue is exposed, a preparation of stems could not be expected to be a good medium for bacterial growth, and this might be

the reason why no inhibitor was produced. Experiments were done in which populations of clean stems were cut, washed, and aerated in sea water. Such preparations did not support the growth of significant numbers of bacteria, and, when tested on stems, permitted regeneration at the same rate as the controls.

A summary of the experiments which have been described, together with the number of cases of each type, is presented in Table III. Cases in which very slight bacterial growth occurred in the preparations or in which the preparations only produced a slight delay in regeneration (such as case B4, Table II) are recorded as negative (−) in the table; only definite cases of bacterial growth or regeneration inhibition are recorded as positive (+). As the table indicates, the inhibition of regeneration was always correlated with the growth of bacteria.

It is pertinent to mention certain experiments done with the regeneration inhibitor found in *Tubularia* hydranth extracts. Such extracts were prepared by homogenizing a population of adult hydranths and collecting the supernatant, as described by Tardent (1955) and Tweedell (1958). It was found that the inhibition of regeneration produced by such extracts was not a result of bacterial growth, in that when penicillin and streptomycin were added to the extracts to maintain bacteriostasis the activity of the extracts was not affected in terms of the proportion of stems inhibited by a given dilution of extract. It was found, however, that in contrast to the original report of Tardent (1955), the inhibition produced by *Tubularia* tissue extracts was not specific to hydranth tissue. The supernatant of homogenates from equivalent quantities of stem tissue also suppressed the regeneration of stems. Tardent (personal communication) has obtained the same result recently with *Tubularia larynx*. Preliminary comparisons on a wet weight basis indicate that hydranth tissue is about twice as active a source of inhibitor as stem tissue. The lack of specificity of this inhibitor makes it impossible, however, in the absence of further data, to adequately evaluate the normal physiological role of the substances involved.

Discussion

The results of the experiments with inhibitor water may be summarized as follows. (1) Hydranths agitated in sea water produce bacterial growth and inhibitors of regeneration. (2) If bacterial growth is suppressed with antibiotics, regeneration inhibitors cannot be collected. (3)If antibotics are added at the beginning of aeration but bacterial growth is not prevented, inhibitors can be collected. (4) Bacterial growth in the absence of hydranths produces regeneration inhibitors. These results, together with the appropriate controls, demonstrate that inhibitor water as prepared in these experiments is a by-product of bacterial growth for which the hydranths serve as inoculum and nutrient source. The results, however, should not be taken to indicate that hydranths cannot produce any inhibitors of regeneration, but rather that inhibitor water prepared as described by previous workers contained no inhibitors which could not be accounted for as the products of bacterial rather than hydranth metabolism.

If hydranths are agitated with antibiotics at densities several-fold higher than those used to prepare inhibitor water (*cf.* Tweedell, 1958), occasionally such preparations (*e.g.*, Table II, B4) retard regeneration even though bacteriostasis has been maintained with antibiotics. It is interesting to note that in such cases

bulbous outgrowths appear at one or both ends of many of the stems. These outgrowths are similar to those found in stems placed in *Tubularia* tissue extracts (Tweedell, 1958; author's unpublished observations), suggesting that the cytolysis of some of the hydranth tissue has released the substances found in hydranth extract into the water.

Since this manuscript was originally submitted for publication, a paper by Tweedell (1958) has appeared in which the results described in the present paper are discussed. The results of this work were presented incompletely by Tweedell; the results as presented here answer the objections raised in his discussion. In particular, the possibility that the antibiotics used had significant effects other than that of maintaining bacteriostasis has been excluded by the results described above.

Tweedell notes that although bacteria were removed from some of his preparations by sterile filtration the preparations still inhibited regeneration. It is clear from the present work that it is not the bacteria themselves, but rather the metabolites they release into the medium, which are primarily responsible for the activity of inhibitor water. Removal of the bacteria from inhibitor water or proteose-peptone solutions after aeration by filtration or centrifugation, or the addition of penicillin and streptomycin to such preparations, in some cases reduced the inhibitory activity of the preparation but in no case eliminated it.

Summary

1. Rose and Rose (1941) found that adult *Tubularia* hydranths agitated in sea water produced a solution, inhibitor water, which prevented regeneration. They and subsequent workers have ascribed to this inhibitor a role in normal physiological dominance. In the present investigation it has been found that considerable bacterial growth occurs in the solution during the preparation of inhibitor water by the usual methods, and that when antibiotics have been added to maintain bacteriostasis no inhibitor can be collected. Experiments have excluded the possibilities that the antibiotics used are preventing the production of the inhibitor or destroying it as it is produced. It has been shown that metabolites produced by bacterial growth in the absence of hydranths inhibit regeneration.

2. These data lead to the conclusion that inhibitor water represents the by-products of bacterial growth for which the hydranths serve as source of inoculum and as nutritive medium.

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